ISSN 1346–7565 Acta Phytotax. Geobot. 57 (1): 95–106 (2006)

Cytological and Genetic Study of Two Putative Hybrids and Their Parents of *Athyrium* (Woodsiaceae; Pteridophyta) in Yakushima Island, Southwestern Japan

YASUKO TERADA[1]* and MASAYUKI TAKAMIYA[2]

*[1]Kumamoto City Museum, 3-2 Furukyo, Kumamoto 860-0007, Japan: [2]Department of Environmental Science,
Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami,
Kumamoto 860-8555, Japan*

The mitotic chromosome number, meiotic behavior and sporogenesis, allozyme variation, chloroplast DNA type, and morphological characteristics of two putative hybrids and their putative parents of *Athyrium* in Yakushima Island, southwestern Japan, were studied. *Athyrium* ×*flavosorum* is a sterile hypotetraploid ($2n = 159$), and its putative parents *A. arisanense* and *A. subrigescens* are a sexually reproductive hypotetraploid ($2n = 158$) and a sexual tetraploid ($2n = 160$), respectively. Allozyme and morphological analyses showed that, *A.* ×*flavosorum* displayed intermediate characteristics between *A. arisanense* and *A. subrigescens*. The chloroplast DNA analysis showed that, *A. arisanense* and *A.* ×*flavosorum* had the same SSCP-PCR banding pattern. *Athyrium* ×*purpurascens* is a sterile tetraploid ($2n = 160$), and its putative parents, *A. subrigescens* and *A. kuratae*, are sexually reproductive tetraploids ($2n = 160$). Allozyme and morphological analyses revealed that *A.* ×*purpurascens* was an intermediate between *A. subrigescens* and *A. kuratae*; the chloroplast DNA of *A.* ×*purpurascens* was the same as that of *A. kuratae*. These data did not contradict the hypothesis that *A.* ×*flavosorum* originated from a hybridization between *A. arisanense* and *A. subrigescens*, whereas *A.* ×*purpurascens* originate from a hybridization between *A. kuratae* and *A. subrigescens*.

Key words: allozyme, *Athyrium*, chloroplast DNA, chromosome, hybrid, Yakushima Island

The genus *Athyrium* includes about 200 taxa distributed worldwide, and the center of species diversity is probably a temperate region in eastern Asia (Iwatsuki 1992). In Japan, 109 *Athyrium* taxa, including 74 putative hybrids, are known, mainly in the southwestern part (Kurata & Nakaike 1990, 1994, Iwatsuki 1992). These putative hybrids have hereto been recognized only base on their morphological characteristics, and there have been no cytological and genetic studies conducted on *Athyrium* to date, with the exception of a single paper concerning the meiotic chromosomes of five putative hybrids of *Athyrium* (Hirabayashi 1970).

Yakushima Island, located in the southwestern part of Japan (30°N, 131°E), has an area of 503 km^2. To date, 44 *Athyrium* taxa (20 species including five endemics, one variety, seven forms, and 16 putative hybrids including 12 endemics) have been recorded in Yakushima Island (Hatusima 2004), which is the area richest in *Athyrium* in Japan; therefore it is a suitable site for the study of *Athyrium*. In recent years, many *Athyrium* taxa recorded in Yakushima Island have become extinct, making it necessary to conduct an immediate taxo-

---

* Present address: 14-8-1-603 Takagi, Fukui 910-0806, Japan

TABLE 1. Materials of *Athyrium* plants used for this study.

Population Code No.	Taxon and Locality[a]	Voucher (*T & T #*)
	***A. arisanense* (Hayata) Tagawa**	
1.	Mt. Mochomu-dake, Yaku-cho, Kumage-gun, Kagoshima Pref.	*409, 410, 412, 414-417, 457-469 & 470*
	***A. subrigescens* (Hayata) Hayata ex H. Itô**	
1.	Mt. Mochomu-dake, Yaku-cho, Kumage-gun, Kagoshima Pref.	*411, 413, 473-475 & 476*
2.	Yakusugi Land, Yaku-cho, Kumage-gun, Kagoshima Pref.	*397, 646 & 647*
3.	Mt. Aiko-dake, Kamiyaku-cho, Kumage-gun, Kagoshima Pref.	*642*
4.	Shiratani-unsuikyou, Kamiyaku-cho, Kumage-gun, Kagoshima Pref.	*400-403 & 406*
5.	Kotsuru, Itsuki-mura, Kuma-gun, Kumamoto Pref.	*138, 139, 421-436 & 437*
	***A. kuratae* Seriz.**	
6.	Okujyusou-keikoku, Ookuchi City, Kagoshima Pref.	*606*
7.	Mt. Tetsu-yama, Ebino City, Miyazaki Pref.	*118 & 119*
5.	Kotsuru, Itsuki-mura, Kuma-gun, Kumamoto Pref.	*140 & 141*
8.	Ookawa, Minamata City, Kumamoto Pref.	*38, 215, 217, 218, 221, 222, 224 & 392*
9.	Imamura, Yamae-mura, Kuma-gun, Kumamoto Pref.	*584*
10.	Yuzukimotomura, Maetsue-mura, Hita-gun, Ooita Pref.	*125*
11.	Nogouchi-keikoku, Sawara-ku, Fukuoka City, Fukuoka Pref.	*167, 168 & 169*
12.	Gosyo-dani, Nakagawa-cho, Tsukushi-gun, Fukuoka Pref.	*104*
13	Mt. Kishi-dake, Aichi-cho, Higashimastuura-gun, Saga Pref.	*634*
	***A. ×flavosorum* Seriz.**	
1.	Mt. Mochomu-dake, Yaku-cho, Kumage-gun, Kagoshima Pref.	*418 & 419*
	***A. ×purpurascens* (Tagawa) Kurata**	
2.	Yakusugi Land, Yaku-cho, Kumage-gun, Kagoshima Pref.	*394*

[a] Populations of code number 1-4 are at Yakushima Island.

nomic study. We have investigated Yakushima Island many times since 2002, but until now we have been able to discover only five *Athyrium*: *A. arisanense* (Hayata) Tagawa, *A. nakanoi* Makino, *A. subrigescens* (Hayata) Hayata ex H. Itô, *A. ×flavosorum* Seriz., and *A. ×purpurascens* (Tagawa) Sa. Kurata. Because *A. nakanoi* is morphologically very different from the other *Athyrium* taxa (Kurata 1961, Kato 1977), *A. nakanoi* was not included in our present study.

We studied two putative hybrids of *Athyrium* and their putative parents in Yakushima Island and Kyushu, southwestern Japan. *Athyrium ×flavosorum* was described as a natural hybrid between *A. arisanense* and *A. subrigescens*, and *A. ×purpurascens* was described as a natural hybrid between *A. kuratae* Seriz. and *A. subrigescens*. *Athyrium × flavosorum* is endemic to Yakushima Island, and *A. arisanense* occurs only in Taiwan and in Yakushima Island. In this article, we present our study of the mitotic chromosome number, meiotic behavior, allozyme variation, chloroplast DNA type, and morphological characteristics of five *Athyrium* taxa (two putative hybrids and three parent species), and demonstrate the hybridity of *A. subrigescens*, and *A. ×purpurascens*.

Materials and Methods

Plant materials

A total of 78 individual plants representing five Japanese *Athyrium* taxa; *A. arisanense* (21 plants), *A. subrigescens* (34), *A. kuratae* (20), *A. ×flavosorum* (2), and *A. ×purpurascens* (1) were examined. The plant names, localities, population code numbers, and vouchers are given in Table 1. The voucher numbers are Terada & Takamiya's collection numbers and are abbreviated as"*T&T #*". Voucher specimens were deposited in the herbarium of Kumamoto University (KUMA). At Mt. Mochomu-dake (population 1), *A. arisanense*, *A. subrigescens*, and *A.* ×*flavosorum* grew side by side in the same population. In Yakusugi Land (population 2), *A. subrigescens* intermingled with *A.* ×*purpurascens*. The identification of the specimens followed the procedure of Kato (1995).

Cytological and reproductive analysis

One to five plants were examined for each taxon. Live study materials were maintained in cultivation in the Botanical Garden, Faculty of Science, Kumamoto University. For the observation of mitotic and meiotic chromosomes, the techniques described in Takamiya (1993) were employed. For the examination of mitotic chromosomes, root tips were pretreated with 0.002 M 8-hydroxyquinoline for 6 h at approximately 20°C. After fixation in 45% acetic acid at about 4°C for 15 min, the root tips were hydrolyzed in 1 N HCl at 60°C for 15 sec and then squashed in 2% aceto-orcein. For the examination of meiotic chromosomes and sporogenesis, young sporangia were fixed with modified Carnoy's solution (absolute ethanol : acetic acid : chloroform = 2 : 1 : 1) overnight at approximately 4°C, refixed with acetic alcohol (absolute ethanol : acetic acid = 3 : 1) at approximately 4°C for 1 h, and then squashed in 2% aceto-orcein.

Allozyme analysis

The genetic structures of the plants were analyzed based on allozyme polymorphisms. Except for *Athyrium kuratae T&T 104*, all plants sampled for this study were used in the allozyme analysis (77 plants examined in total). Fresh leaves were ground in cold extraction buffer, as described by Murakami *et al.* (1999), and the enzymes were resolved on 7.5% polyacrylamide gels, following the procedures of Shiraishi (1988). Aspartate amino transferase (AAT, E. C. 2. 6.1. 1), Hexokinase (HK, E. C. 2. 7. 1. 1), Isocitrate dehydrogenase (IDH, EC 1.1.1.42), Phosphoglucoisomerase (PGM, EC 2.7.5.1), and Triose-phosphate isomerase (TPI, EC 5.3.1.1) were analyzed following the procedure of Shiraishi (1988).

PCR-SSCP analysis of chloroplast DNA

The chloroplast DNAs were analyzed based on single-strand conformation polymorphisms (SSCP) following the procedures of Watano *et al.* (1995). Except for Imamura (population 9), one to six plants collected from each population were used for the PCR-SSCP (29 plants examined in total). Total DNA was extracted from dry leaves by the CTAB isolation method (Doyle & Doyle 1987). Amplifications of the intergenic spacer region between *psb*C-*trn*S (UGA) of the cpDNA were carried out following the procedure of Nishizawa & Watano (2000). The following cycling profile was used: initial 3 min denaturation at 95°C, followed by 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were denatured at 94°C for 3 min, and electrophoresed in 10% polyacrylamide gel at approximately 10°C for 6 h in 350 V, followed by silver staining (Watano *et al.* 1995).

Morphological analysis

Several qualitative morphological characters of the pinna segments were compared across the five taxa, using the voucher specimens.

Results

Chromosomes and reproduction

The results are summarized in Table 2.

The mitotic chromosome number of two *Athyrium arisanense* plants collected from Mt. Mochomu-dake was determined to be $2n = 158$ (Fig. 1A). For four *A. subrigescens* plants collected from three populations, five *A. kuratae* plants from five populations, and one *A.* ×*purpurascens* plant from Yakusugi Land, the mitotic chromosome numbers was found to be $2n = 160$ (Figs. 1B, 1C &

TABLE 2. Number of chromosomes and shape of spores.

Taxon and Population Code No. (see Table 1)	Voucher (*T & T #*)	Chromosome number		Spore[b]
		2n	n	
A. arisanense				
Population 1	*409 & 416*	158	-	N
	417	c158	-	-
A. subrigescens				
Population 1	*411*	160	-	-
Population 4	*400*	160	-	-
Population 5	*138*	160	80II	N
	139	160	-	N
A. kuratae				
Population 5	*141*	160	-	-
Population 6	*606*	c160	-	-
Population 8	*38*	160	80II	N
Population 11	*167*	160	-	-
Population 12	*104*	160	-	-
A. ×flavosorum				
Population 1	*418*	159	-	Ab
	419	159	Irr[a]	Ab
A. ×purpurascens				
Population 2	*394*	160	Irr[a]	Ab

[a] Irr = Irregular meiosis.

[b] N = normally shaped spores. Ab = abnormally shaped spores.

1D). For two *A. ×flavosorum* plants collected from Mt. Mochomu-dake, the mitotic chromosome number was determined to be $2n = 159$ (Fig. 1E).

At the first meiotic metaphase, 80 bivalents were observed in *Athyrium subrigescens* and *A. kuratae*; subsequent meiotic behavior was normal, and finally 64 normally shaped spores were produced per sporangium (Figs. 3A & 3B). In *A. arisanense*, 64 normally shaped spores were produced per sporangium (Fig. 3E). The meiotic behaviors of *A. ×flavosorum* and *A. ×purpurascens* were irregular, sporogenesis was irregular, and ultimately 16 to approximately 70 abnormally shaped spores were produced per sporangium (Figs. 3C, 3D & 3F).

Allozyme variations

Using five enzyme systems, seven loci, *Aat*, *Pgm-1*, *Pgm-2*, *Idh*, *Hk*, *Tpi-1*, and *Tpi-2*, were interpreted in the five *Athyrium* taxa. The locus *Pgm-2* showed no variation throughout the materials examined. Two alleles (*a* & *b*) were observed at *Pgm-2* and *Idh*. Three alleles (*a-c*) were observed at *Pgm-1*, *Tpi-1*, and *Tpi-2* (Fig. 5). Four (*a-d*) and five alleles (*a-e*) were observed at *Aat* and *Hk*, respectively (Fig. 5).

Based on the combination of genotypes at two polymorphic loci, *Aat* and *Tpi-2*, seven multi-locus genotypes were recognized in 21 *Athyrium arisanense* plants from one population. Concerning the two polymorphic loci, *Aat* and *Pgm-1*, four multi-locus genotypes were recognized in 19 *A. kuratae* plants from eight populations. There were no allozyme variations in 34 *A. subrigescens* plants from five populations and two *A. ×flavosorum* plants from one population (Table 3).

PCR-SSCP analysis of chloroplast DNA

Three types of banding patterns were observed for the five *Athyrium* taxa, and the patterns were named type I, II, and III (Fig. 6). There were no intraspecific variations in the banding patterns of *A. arisanense*, *A. subrigescens*, and *A. kuratae*. The banding patterns of six *A. arisanense* plants collected from one population, ten *A. subrigescens* plants from five populations, and ten *A. kuratae* plants from eight populations were recognized as type I, type II, and type III, respectively. Two *A. ×flavosorum* plants collected from one population and one *A. × purpurascens* plant displayed a type I and a type III banding pattern, respectively (Table 3).

Morphological characteristics of the pinna segments

The pinna segments of *Athyrium arisanense* were serrate at the margin and were decurrent to narrow costal wings (Figs. 7A & 7B). *Athyrium subrigescens* had fully pinnate pinnae, obvious pinnules with short stalks, and a serrate to dentate margin (Figs. 7E & 7F). The pinna segments of *A. kuratae* were entire at the margin and were decurrent to costae (Figs. 7I & 7J). The morphological characteristics of the pinna segments of *A. ×flavosorum*

TABLE 3. Summary of phenotypes of alleles at seven loci and types of chloroplast DNA.

Taxon	Locus							Type of DNA
	Aat	*Pgm-1*	*Pgm-2*	*Idh*	*Hk*	*Tpi-1*	*Tpi-2*	
A. arisanense	*abbb* (1)[a] *bbbb* (5) *bbcc* (7) *bbdd* (8)	*bbcc* (21)	*aabb* (21)	*aabb* (21)	*aabb* (21)	*bbbb* (21)	*accc* (15) *aacc* (6)	I (6)
A. subrigescens	*bbbb* (34)	*bbbb* (34)	*aabb* (34)	*aaaa* (34)	*ccee* (34)	*aacc* (34)	*bbcc* (34)	II (10)
A. kuratae	*aabb* (12) *abbb* (5) *bbbb* (2)	*bbbb* (18) *aabb* (1)	*aabb* (19)	*aaaa* (19)	*bbdd* (19)	*bbbb* (19)	*cccc* (19)	III (10)
A. ×*flavosorum*	*bbbc* (2)	*bbbc* (2)	*aabb* (2)	*aaab* (2)	*abce* (2)	*abbc* (2)	*abcc* (2)	I (2)
A. ×*purpurascens*	*abbb* (1)	*bbbb* (1)	*aabb* (1)	*aaaa* (1)	*bcde* (1)	*abbc* (1)	*bccc* (1)	III (1)

[a]( ) = number of plant

and *A.* ×*purpurascens* were intermediate between those of *A. arisanense* and *A. subrigescens* (Figs. 7C & 7D) and between those of *A. kuratae* and *A. subrigescens* (Figs. 7G & 7H), respectively.

Discussion

Cytology

The chromosome numbers for *Athyrium arisanense* and *A. kuratae* have been known. Tsai & Shieh (1983) reported that the chromosome number for *A. arisanense* is $n = 82$, based on a plant collected from Tienchih in Taiwan; subsequently (1985), they determined 40 tetravalents at the meiotic metaphase in plants from the same place. Concerning their microphotograph of the meiotic chromosomes of *A. arisanense* (Fig. 66 in Tsai & Shieh 1983), we are able to count $n = 40$, not 82. There were no photographs and/or drawings in the subsequent paper by Tsai & Shieh (1985). Thus, the chromosome number for Taiwanese *A. arisanense* is uncertain, and a reexamination is required. Regardless of the accuracy of the previously reported chromosome number for Taiwanese *A. arisanense*, our count of the mitotic chromosome number of $2n = 158$ is new for *A. arisanense*. Hirabayashi (1970) determined the chromosome number for *A. kuratae* is $n = 80$, based on a plant collected from Kusenbu, Saga Prefecture. The mitotic chromosome number of $2n = 160$, determined based on five *A. kuratae* plants, confirms Hirabayashi's result. The chromosome numbers for *A. subrigescens*e, *A.* ×*flavosorum*, and *A.* ×*purpurascens* are reported here for the first time.

The basic chromosome numbers for genus *Athyrium* have been reported as $x = 40$ or $x = 41$ (Chiarugi 1960, Fabbri 1963, 1965, Löve *et al.* 1977, Goldblatt 1981, 1984, 1985, 1988, Goldblatt & Johnson 1990, 1991, 1994, 1996, 1998, 2000, 2003). Because most species with $x = 41$ belonging to the genus *Athyrium* have been moved to the genus *Diplazium*, a sister clade of *Athyrium*, as a result of a molecular phylogenetic study (Sano *et al.* 2000), $x = 40$ is the commonly accepted basic chromosome number for *Athyrium*, with the exception of three species which have $x = 41$ (Bhavanandan 1968, Srivastava 1985, Bhavanandan & Ammal 1991). Nakato (1988) determined that *A. nikkoense* Makino has chromosome number of $2n = 78$, and this is the only report of aneuploidy from $x = 40$ to $x = 39$. Because *A. subrigescens* and *A. kuratae* were found to have $2n = 160$ and 80 bivalents at metaphase I and displayed normal sporogenesis, they are assumed to be sexually reproductive tetraploids based on $x = 40$. The mitotic chromosome number of $2n = 158$ for *A. arisanense* is new for the genus *Athyrium*, and should be the result of

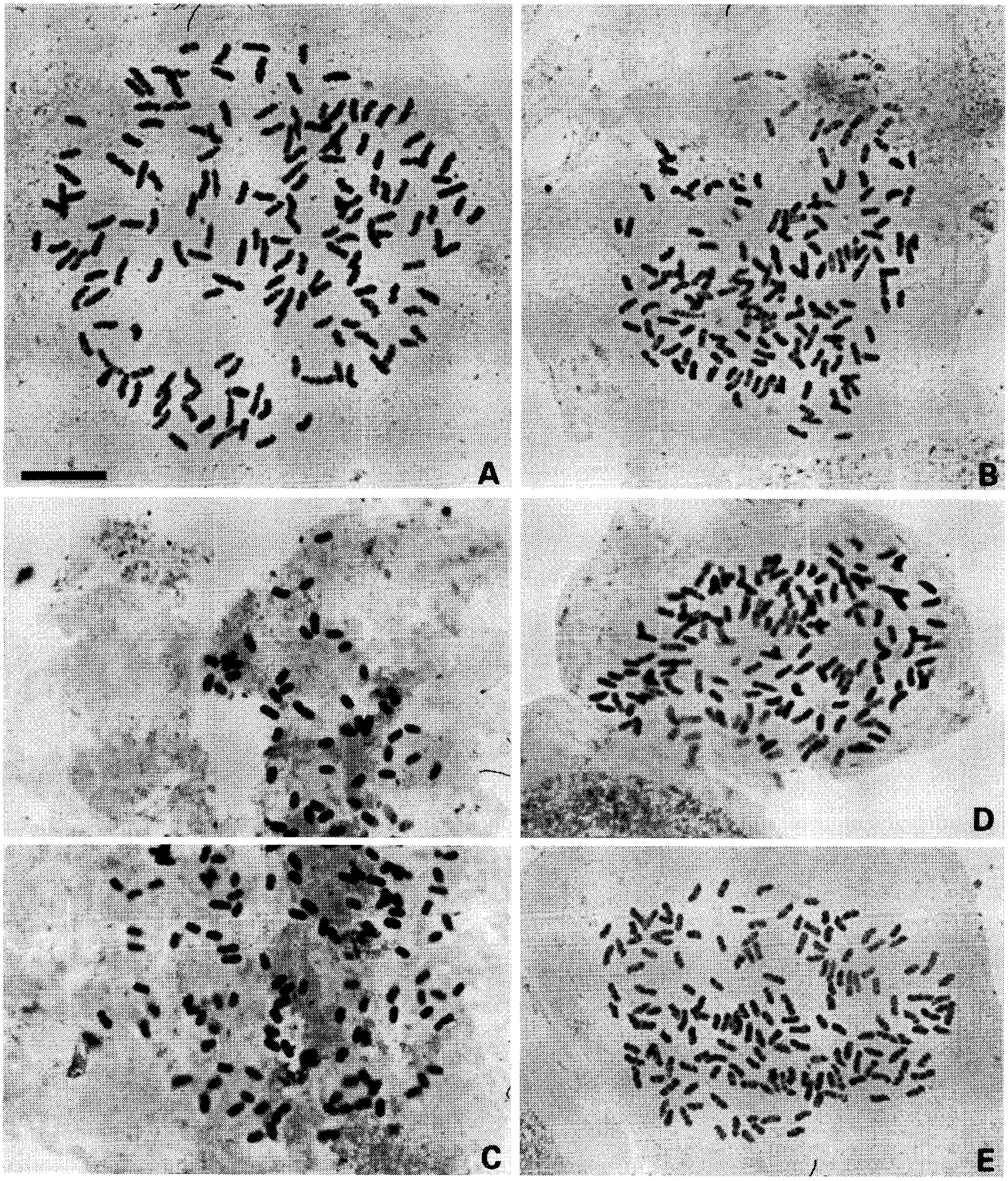

FIG. 1. Mitotic metaphase chromosomes of *Athyrium* plants. A, *A. arisanense*, $2n = 158$ (*T&T 409*); B, *A. subrigescens*, $2n = 160$ (*T&T 411*); C, *A. kuratae*, $2n = 160$ (*T&T 38*); D, *A.* ×*purpurascens*, $2n = 160$ (*T&T 394*); E, *A.* ×*flavosorum*, $2n = 159$ (*T&T 418*). Bar = 10µm.

aneuploidy from $2n = 160$. Since *A. arisanense* produces normally shaped spores, it is a sexually reproductive hypotetraploid. The putative hybrids of *A.* ×*flavosorum* ($2n = 159$) and *A.* ×*purpurascens* ($2n = 160$) displayed irregularities throughout meiosis and sporogenesis. Thus, *A.* ×*flavosorum* is a sterile hypotetraploid and *A.* ×*purpurascens* is a sterile tetraploid.

According to Kurata (1961, 1965, 1969) and Serizawa (1970, 1985), the five taxa examined in this study belong to the *Athyrium otophorum* group which includes 23 Japanese taxa (nine species, one

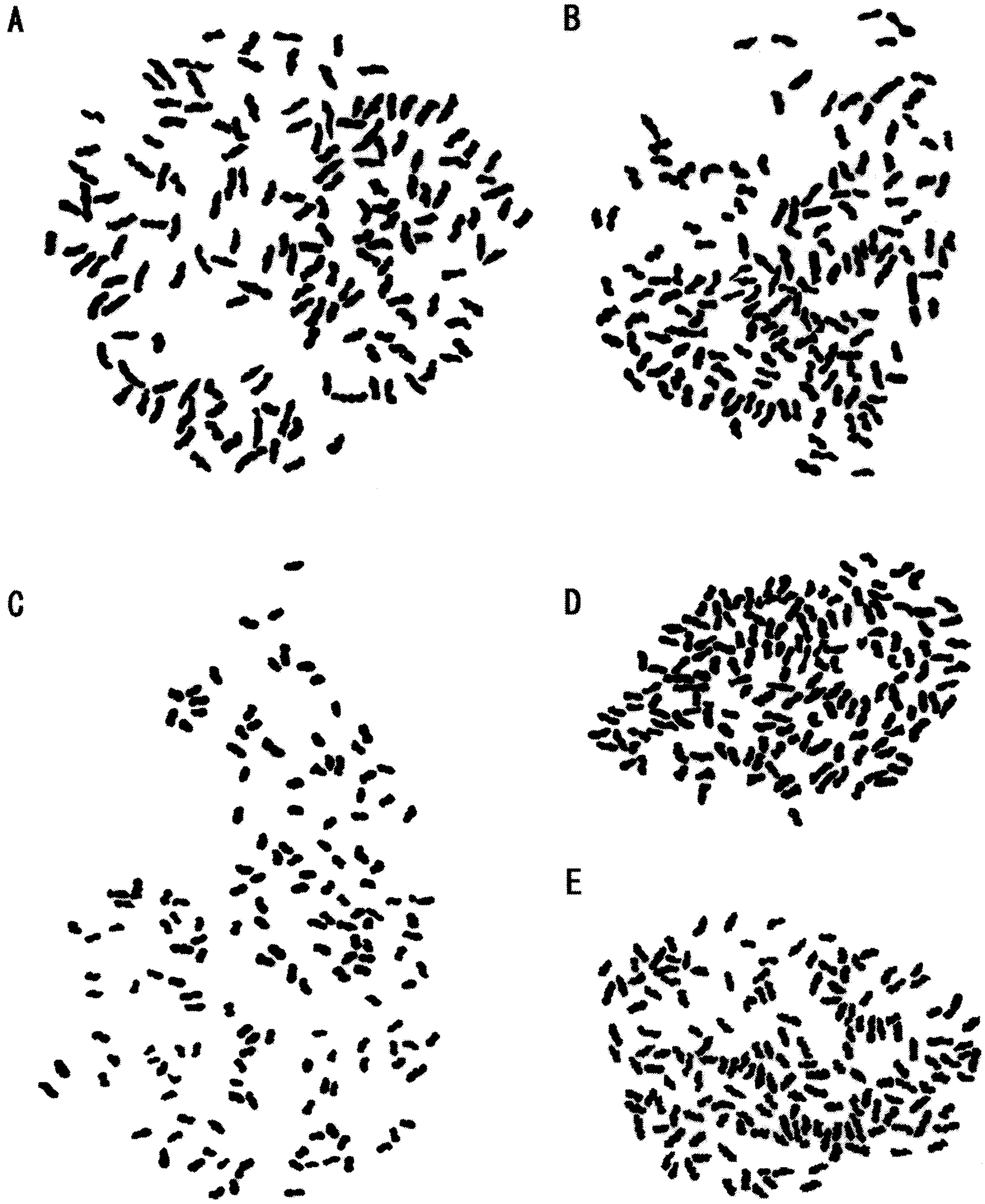

FIG. 2. Explanatory drawings. Figs. A, B, C, D, and E correspond to Figs. 1A, 1B, 1C, 1D, and 1E, respectively.

variety, two forms and eleven putative hybrids). Out of those 23 taxa, the chromosome numbers of twelve taxa have been reported in previous and present studies; eleven of them are tetraploids (Takamiya 1996), while one, *A. oblitescens* is an intraspecific polypoid with tetraploids and hexaploids (Kurihara *et al.* 1996). Thus, the species of the *A. otophorum* group seem to have evolved at the tetraploid level.

Hybridity of Athyrium ×flavosorum

Athyrium ×flavosorum was described as a natural hybrid between *A. arisanense* and *A. subrigescens* (Serizawa 1985). No cytological data have been collected for this putative hybrid previously. The present cytological data show that *A. ×flavosorum* is

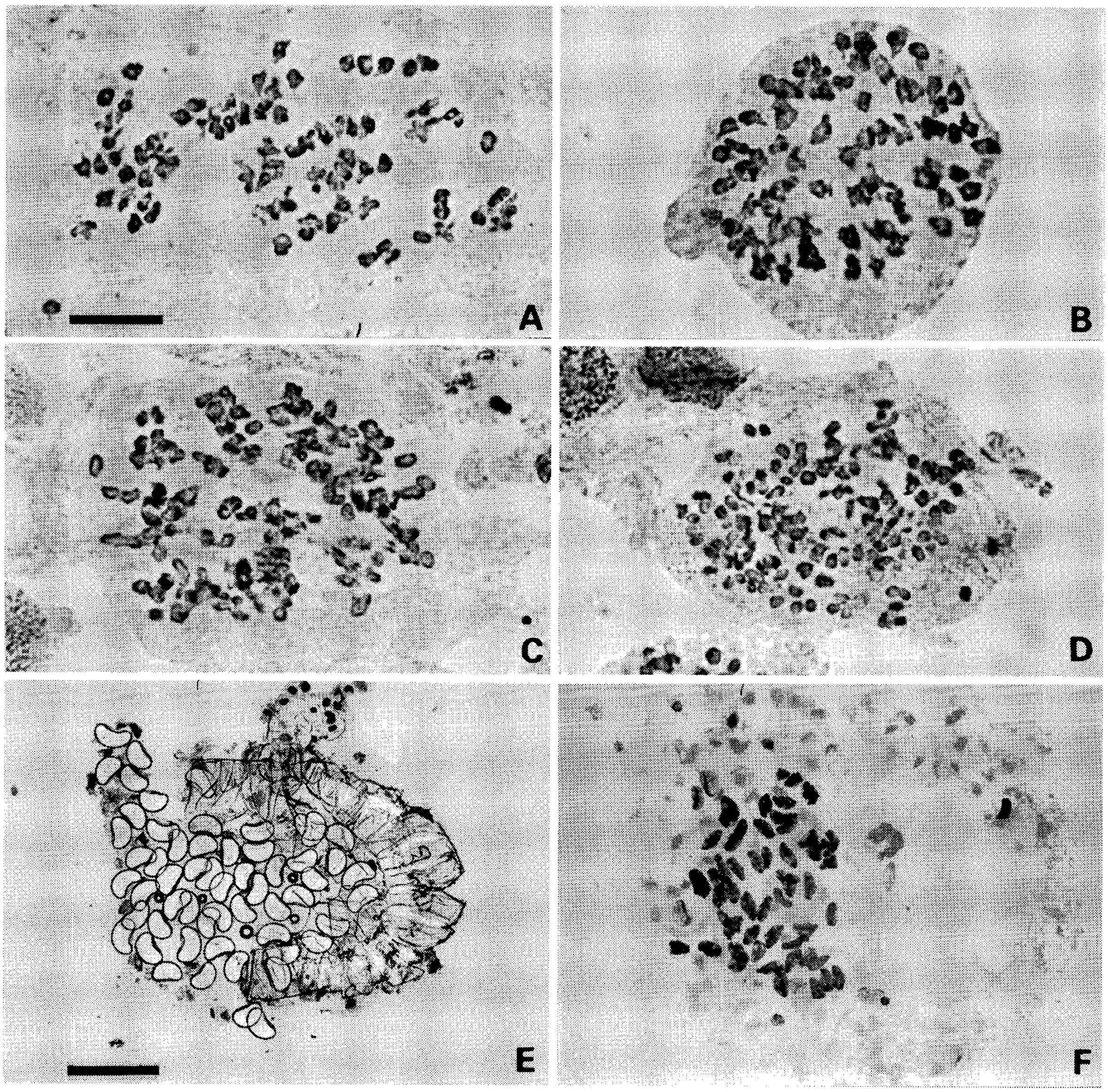

FIG. 3. Meiotic chromosomes at metaphase I and spores of *Athyrium* plants. A, *A. subrigescens*, $n = 80\mathrm{II}$ (*T&T 138*); B, *A. kuratae*, $n = 80\mathrm{II}$ (*T&T 38*); C, *A.* ×*flavosorum*, irregular, approximately 50II+60I (*T&T 418*); D, *A.* ×*purpurascens*, irregular, approximately 30II+100I (*T&T 394*); E, normally shaped spores of *A. arisanense* (*T&T 409*); F, abnormally shaped spores of *A.* ×*flavosorum* (*T&T 419*). Bar = 10μm at Figs. A-D, and 100μm at Figs. E and F.

a sterile hypotetraploid with $2n = 159$. Because the putative parents *A. arisanense* and *A. subrigescens* have $2n = 158$ and $2n = 160$, respectively, the hybrid origin of *A.* ×*flavosorum* was not contradicted cytologically.

Our allozyme analysis showed that the multilocus genotype of *Athyrium* ×*flavosorum* is *Aat*^{bbbc}, *Pgm-1*^{bbbc}, *Pgm-2*^{aabb}, *Idh*^{aaab}, *Hk*^{abce}, *Tpi-1*^{abbc}, and *Tpi-2*^{abcc}. Because the alleles *a* at *HK* and *b* at *Tpi-2* are unique, species-specific alleles for *A. arisanennse* and *A. subrigescens*, respectively, the alleles *Hk*^{a} must be inherited from *A. arisanense* and *Tpi-2*^{b} from *A. subrigescens*. The genotype of *A.* ×*flavosorum* can be created by adding a half set of the *A. arisanense* genotype (*Aat*^{bc}, *Pgm-1*^{bc}, *Pgm-2*^{ab}, *Idh*^{ab}, *Hk*^{ab}, *Tpi-1*^{bb}, and *Tpi-2*^{ac}) to a half set of the *A. subrigescens* genotype (*Aat*^{bb}, *Pgm-1*^{bb}, *Pgm-2*^{ab}, *Idh*^{aa}, *Hk*^{ce}, *Tpi-1*^{ac}, and *Tpi-2*^{bc}). Our morphological analysis also showed that *A.* ×*flavosorum* is an intermediate between *A. arisanense* and *A. subrigescens*.

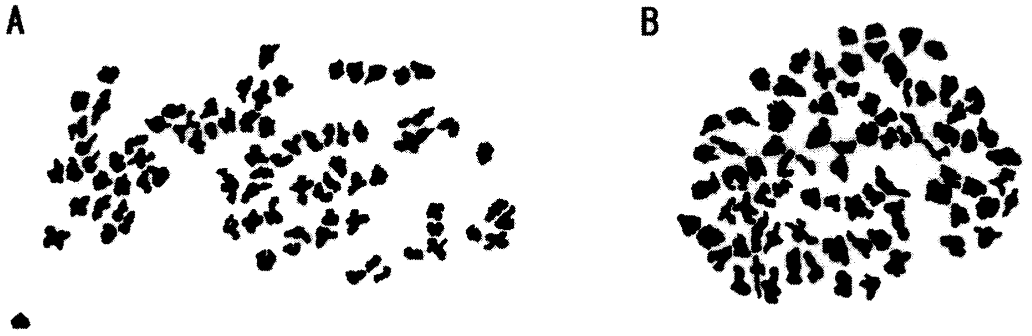

FIG. 4. Explanatory drawings. Figs. A and B correspond to Figs. 3A and Fig. 3B, respectively.

Thus, the allozyme and morphological data did not contradict the hypothesis that *A.* ×*flavosorum* originated from a hybridization between *A. arisanense* and *A. subrigescens*, either. Furthermore, our analysis of the chloroplast DNA showed that *A.* ×*flavosorum* and *A. arisanense* have a common type I banding pattern, whereas *A. subrigescens* has a type II pattern. Because the chloroplast DNA of ferns is inherited maternally (Gastony & Yatskievych 1992), the mother species of the two *A.* ×*flavosorum* individuals examined in this study must be *A. arisanense*.

Hybridity of Athyrium ×purpurascens

Athyrium ×*purpurascens* was described as a natural hybrid between *A. subrigescens* and *A. kuratae* (Kurata 1965). No cytological data have been collected for this putative hybrid previously. Because the putative parents *A. subrigescens* and *A. kuratae*, are sexual tetraploids ($2n = 160$), the hybrid origin of *A.* ×*purpurascens* (a sterile tetraploid with $2n = 160$) between these two taxa was not contradicted cytologically.

The allozyme multi-locus genotype of *Athyrium* ×*purpurascens* was shown to be Aat^{abbb}, $Pgm\text{-}1^{bbbb}$, $Pgm\text{-}2^{aabb}$, Idh^{aaaa}, Hk^{bcde}, $Tpi\text{-}1^{abbc}$, and $Tpi\text{-}2^{bccc}$. Because the allele *b* at *Tpi-2* is species-specific to *A. subrigescens*, as mentioned above, one of the parents of *A.* ×*purpurascens* must be *A. subrigescens*. In all of the *A. subrigescens* plants examined, the locus *Hk* displayed a fixed heterozygosity of *bbdd*. The alleles at *Hk* of *A.* ×*purpurascens* were *bcde*, which means that the other parent of *A.* ×*purpurascens* must have both a *c* and an *e* allele at *Hk*. Because *A. kuratae* had a fixed heterozygosity of *ccee* at *Hk*, the other parent of *A.* ×*purpurascens* must be *A. kuratae*. Also the multi-locus genotype of *A.* ×*purpurascens* can be created by adding a half set of the *A. subrigescens* genotype (Aat^{bb}, $Pgm\text{-}1^{bb}$, $Pgm\text{-}2^{ab}$, Idh^{aa}, Hk^{ce}, $Tpi\text{-}1^{ac}$, and $Tpi\text{-}2^{bc}$) to a half set of the *A. kuratae* genotype (Aat^{ab}, $Pgm\text{-}1^{bb}$, $Pgm\text{-}2^{ab}$, Idh^{aa}, Hk^{bd}, $Tpi\text{-}1^{bb}$, and $Tpi\text{-}2^{cc}$). Furthermore, several morphological characteristics of *A.* ×*purpurascens* were found to be intermediate between those of *A. subrigescens* and those of *A. kuratae*. Thus, the allozyme data and the morphological data did not contradict the hypothesis that *A.* ×*purpurascens* originated from a hybridization between *A. subrigescens* and *A. kuratae*, either. Because the chloroplast DNA of *A.* ×*purpurascens* and *A. kuratae* displayed the same type III banding pattern, the mother species of the *A.* ×*purpurascens* individual examined in this study must be *A. kuratae*.

Conservation of Athyrium arisanense *as a threatened species*

To date, 44 taxa of *Athyrium* have been recorded in Yakushima Island. Nowadays, the numbers of individuals and populations of many *Athyrium* species are drastically reduced, several species become

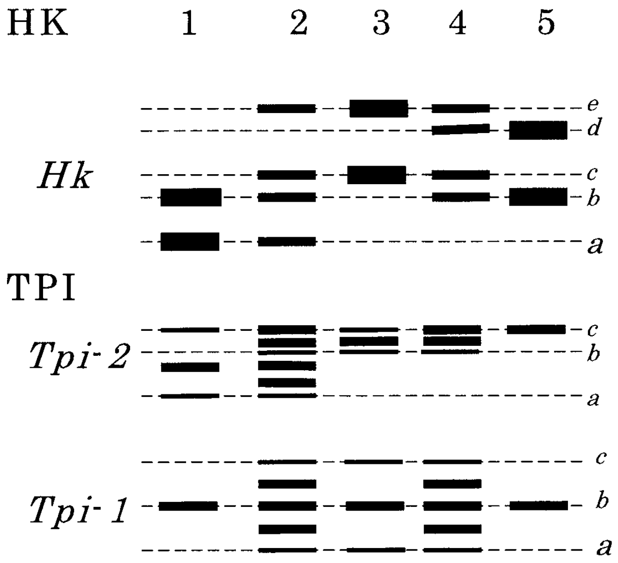

FIG. 5. Schematic explanations for representative electrophoretic banding patterns of HK and TPI. Lanes 1, *Athyrium arisanense* (*T&T 409*); 2, *A.* ×*flavosorum* (*T&T 418*); 3, *A. subrigescens* (*T&T 406*); 4, *A.* ×*purpurascens* (*T&T 394*); 5, *A. kuratae* (*T&T 38*). Anode = bottom of the drawings, cathode = top of the drawings.

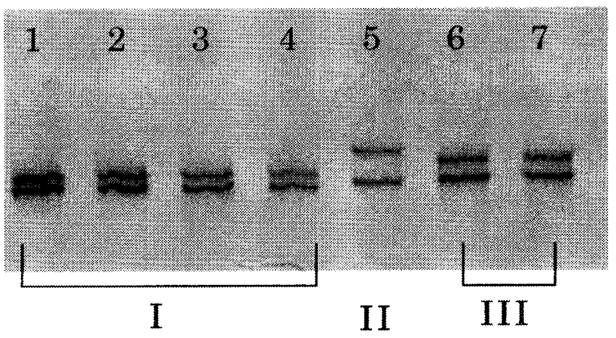

FIG. 6. Representative PCR-SSCP banding patterns of intergenic spacer region between *psb*C-*trn*S of cpDNA. Lanes 1 and 2, *Athyrium arisanense* (*T&T 416, 417*); 3 and 4, *A.* × *flavosorum* (*T&T 418, 419*); 5, *A. subrigescens* (*T&T 413*); 6, *A.* ×*purpurascens* (*T&T 394*); 7, *A. kuratae* (*T&T 141*). Lanes 1-4 are Type I, 5 is Type II, and 6 and 7 are Type III. Anode = bottom of the photograph, cathode = top of the photograph.

extinct, and 16 out of 20 species are listed as threatened species in the Red Data Book of Kagoshima Prefecture (2003). One of the proposed reasons for the decrease is the feeding pressure exerted by sika deer (*Cervus nippon yakushimae*). The population densities of *C. n. yakushimae* in the lowland forest of the western part of Yakushima Island have been estimated at 43-70 and 51.5-63.8 head/km^2 (Agetsuma *et al.* 2003, Tsujino & Yumoto 2004), which might be high enough to result in damage to the natural vegetation.

Athyrium arisanense occurs in Taiwan and Yakushima Island, and is listed as endangered species IA and I in the Red Data Book of Japan (Environment Agency of Japan 2000) and that of Kagoshima Prefecture (Kagoshima Prefecture 2003), respectively. In the present study, we found many *A. arisanense* plants growing at Mt. Mochomu-dake; they constitute the only known *A. arisanense* plant population in Japan. We found that *A. arisanense* has the unique chromosome number of $2n = 158$, and 21 of the plants examined displayed a large allozyme polymorphism. We need to conserve the *A. arisanense* plant population at Mt. Mochomu-dake, and prevent any further damage by sika deer.

We thank Yasuhiko Inoue, Masakatsu Ishizaka, Tadashi Kariyazaki, Katsutoshi Maruno, Hirohumi Tsuji, and Sadao Tsutsui for their assistance in field surveys and providing living materials, Hiroshi Ishikawa and Yasuyuki Watano for teaching a method of PCR-SSCP analysis of chloroplast DNA, and Yakushima Forest Environmental Conservation Center for giving us permission to undertake the research. We are also grateful to the members of our laboratory for maintaining live study materials and to two anonymous reviewers for improvement of a former version of the manuscript. This research was partly funded by RIHN (Research Institute for Humanity Nature) Project (P2-2).

References

Agetsuma, N., H. Sugiura, D. A. Hill, Y. Agetsuma-Yanagihara & T. Tanaka. 2003. Population density and group composition of Japanese sika deer (*Cervus nippon yakushimae*) in an evergreen broad-leaved forest in Yakushima, southern Japan. Ecol. Res. 18: 475-483.

Bhavanandan, K. V. 1968. Studies on the cytology of sixteen species of south Indian ferns. Caryologia 21: 333-338.

—— & L. S. Ammal. 1991. Cytological investigations on family Aspidiaceae (*sensu* Copeland) from south

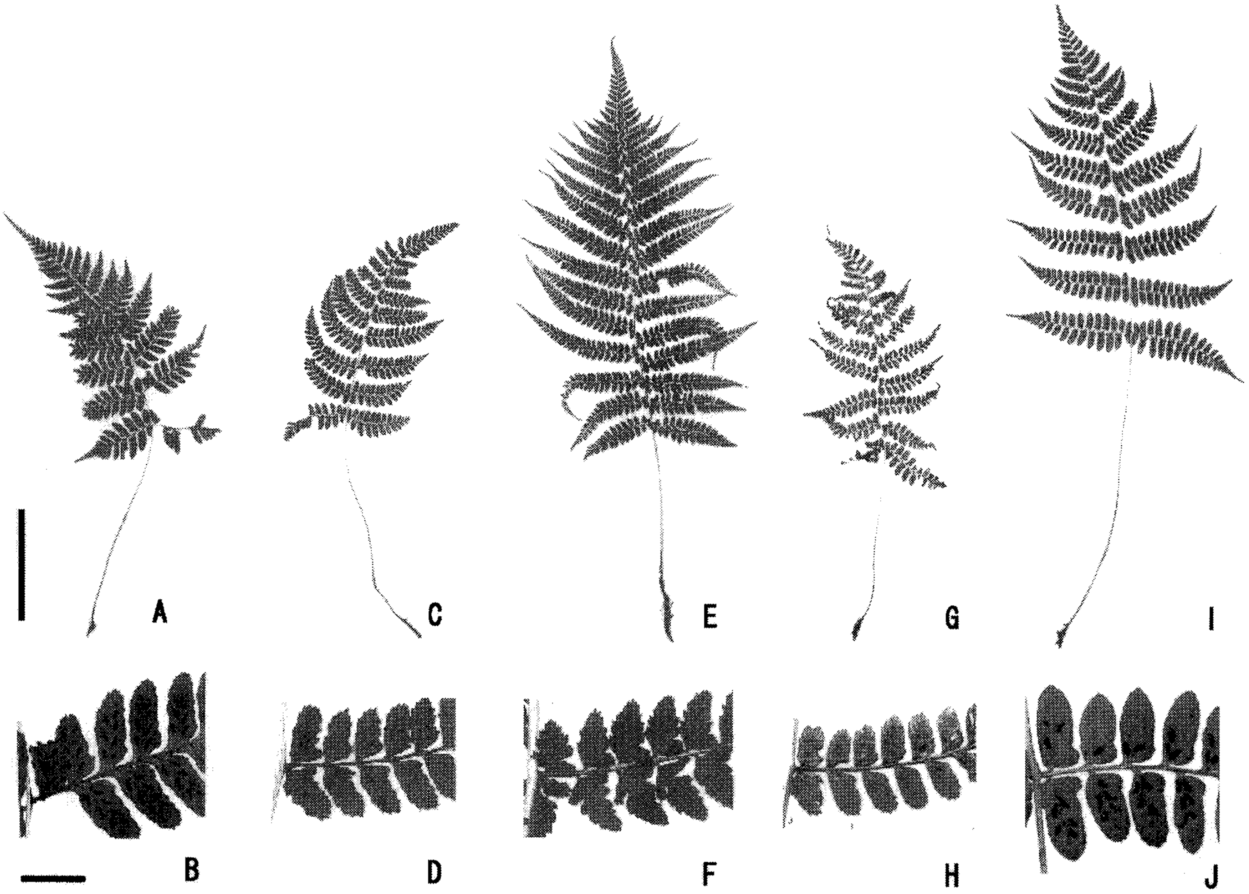

FIG. 7. Leaves and a part of pinnae. A and B, *Athyrium arisanense* (*T&T 414*); C and D, *A.* ×*flavosorum* (*T&T 418*); E and F, *A. subrigescens* (*T&T 406*); G and H, *A.* ×*purpurascens* (*T&T 394*); I and J, *A. kuratae* (*T&T 104*). Bar = 10 cm at leaves, and 1cm at pinnae.

India. Indian Fern J. 8: 78-86.

Chiarugi, A. 1960. Tavole cromosomiche delle Pteridophyta. Caryologia 13: 27-150.

Doyle, J. J. & J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. 19: 11-15.

Environment Agency of Japan. 2000. Threatened Wildlife of Japn. —Red Data Book 2nd ed. -. Japan Wildlife Research Center, Tokyo. (in Japanese)

Fabbri, F. 1963. Primo supplemento alle "Tavole cromosomiche delle Pteridophyta" di Alberto Chiarugi. Caryologia 16: 237-335.

——. 1965. Secondo supplemento alle "Tavole cromosomiche delle Pteridophyta" di Alberto Chiarugi. Caryologia 18: 675-731.

Gastony, G. J. & G. Yatskievych. 1992. Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. Amer. J. Bot. 79: 716-722.

Goldblatt, P. 1981, 1984, 1985, 1988. Index to Plant Chromosome Numbers, Volumes for 1975-1978, 1979-1981, 1982-1983, 1984-1985. Missouri Botanical Garden, St. Louis.

—— & D. E. Johnson. 1990, 1991, 1994, 1996, 1998, 2000, 2003. Index to Plant Chromosome Numbers, Volumes for 1986-1987, 1988-1989, 1990-1991, 1992-1993, 1994-1995, 1996-1997, 1998-2000. Missouri Botanical Garden, St. Louis.

Hatusima, S. 2004. Flora of Kyushu. —A List of Ferns and Flowering Plants from Kyushu including the Amami Islands-. The Kagoshima University Museum, Kagoshima. (in Japanese)

Hirabayashi, H. 1970. Chromosome numbers in several species of the Aspidiaceae (2). J. Jap. Bot. 45: 45-52.

Iwatsuki, K. (ed.) 1992. Ferns and Fern Allies of Japan. Heibonsha, Tokyo. (in Japanese)

Kagoshima Prefecture. 2003. Red Data Book of Kagoshima Prefecture. Kagoshima-ken-kanko-giju-tu-kyokai, Kagoshima. (in Japanese)

Kato, M. 1977. Classification of *Athyrium* and allied genera of Japan. Bot. Mag. Tokyo 90: 13-40.

——. 1995. *Athyrium* Roth. *In*: K. Iwatsuki, T. Yamazaki, D. E. Boufford & F. Ohba, (eds.). Flora of Japan. Vol. I. Pteridophyta and Gymnospermae, Kodansha, Tokyo, pp. 201-213.

Kurata, S. 1961. On the Japanese ferns belonging to the *Athyrium otophorum* group. Sci. Rep. Yokosuka City Mus. 6: 7-26.

——. 1965. Notes on Japanese ferns (37). J. Geobot. 14: 2-6.

——. 1969. Notes on Japanese ferns (46). J. Geobot. 17: 2-5.

—— & T. Nakaike. 1990. Illustrations of Pteridophytes of Japan. Vol. 6. University of Tokyo Press. Tokyo. (in Japanese)

—— & Nakaike. 1994. Illustrations of Pteridophytes of Japan. Vol. 7. University of Tokyo Press. Tokyo. (in Japanese)

Kurihara, T., Y. Watano, M. Takamiya & T. Shimizu. 1996. Electrophoretic and cytological evidence for genetic heterogeneity and the hybrid origin of *Athyrium oblitescens*. J. Plant Res. 109: 29-36.

Löve, Á., D. Löve & R.E.G. Pichi Sermolli. 1977. Cytotaxonomical Atls of the Pteridophyta. J. Cramer, Vaduz.

Murakami, N., M. Watanabe, J. Yokoyama, Y. Yatabe, H. Iwasaki & S. Serizawa. 1999. Molecular taxonomic study and revision of the three Japanese species of *Asplenium* sect. Thamnopteris. J. Plant Res. 112: 15-25.

Nakato, N. 1988. Notes on chromosomes of Japanese pteridophytes (2). J. Jap. Bot. 63: 214-218.

Nishizawa, T. & Y. Watano. 2000. Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. J. Phytogeogr. Taxon. 48: 63-66.

Sano, R., M. Takamiya, M. Ito, S. Kurita & M. Hasebe. 2000. Phylogeny of the lady fern group, tribe Physematieae (Dryopteridaceae), based on chloroplast *rbc*L gene sequences. Mol. Phylogenet. Evol. 15: 403-413.

Serizawa, S. 1970. Taxonomical notes on Asian ferns (1). J. Jap. Bot. 45: 117-122.

——. 1985. Miscellaneous notes on Japanese Pteridophytes (4). J. Phytogeogr. Taxon. 33: 73-77.

Shiraishi, S. 1988. Inheritance of isozyme variations in Japanese black pine, *Pinus thunbergii* Parl. Silvae Genet. 37: 93-100.

Srivastava, R. B. 1985. Ferns of the Indo-Nepal border. Proc. Roy. Soc. Edinburgh, sec. B. Biol. 86: 471.

Takamiya, M. 1993. Chromosome numbers of *Woodsia kitadakensis* and *W. subcordata* (Woodsiaceae). J. Jap. Bot. 68: 73-76.

——. (ed.) 1996. Index to Chromosomes of Japanese Pteridophyta (1910-1996). Japan Pteridological Society, Tokyo.

Tsai, J.-L. & W.-C. Shieh. 1983. A cytotaxonomic survey of the pteridophytes in Taiwan. J. Sci. Eng. 20: 137-158.

——. & ——. 1985. A cytotaxonomic survey of the fern family Aspidiaceae (*sensu* Copeland) in Taiwan. J. Sci. Eng. 22: 121-144.

Tsujino, R. & T. Yumoto. 2004. Effects of sika deer on tree seedlings in a warm temperate forest on Yakushima Island, Japan. Ecol. Res. 19: 291-300.

Watano, Y., M. Imazu & T. Shimizu. 1995. Chloroplast DNA typing by PCR-SSCP in the *Pinus pumila* - *P. parviflora* var. *pentaphylla* complex (Pinaceae). J. Plant Res. 108: 493-499.

Received July 5, 2005; accepted December 26, 2005